Carrot pathogen '*Candidatus* Liberibacter solanacearum' haplotype C detected in symptomless potato plants in Finland

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Abstract

'*Candidatus* Liberibacter solanacearum' (CLso) haplotype C, a bacterial pathogen transmitted by the carrot psyllid *Trioza apicalis*, causes yield losses in carrot production. Due to concerns that this pathogen might also threaten potato (*Solanum tuberosum*) production, the occurrence of CLso in cultivated and volunteer potatoes in Tavastia Proper and Satakunta regions of Finland was studied. Volunteer potato plants were found in 13 of the 27 inspected carrot fields. Of the 148 potato samples tested by PCR, eight volunteer potato plants and one cultivated potato grown at the edge of a carrot field were found CLso positive. The PCR products obtained from these potatoes with primers OA2/OI2c, LpFrag4-1611F/LpFrag4-480R and CL514F/CL514R all showed 100% sequence identity to CLso haplotype C. This is the first observation of CLso haplotype C in field-grown potatoes. In addition, transmission experiments were performed. Attempts to transmit CLso into potato with carrot psyllids were not successful; however, CLso haplotype C was transmitted from infected carrots to potato plants by leaf grafting and by phloem connection formed by dodder, a parasitic plant, and found to survive in the potato plants for several weeks after transmission. However, the bacterial colonization progressed slowly in the potato phloem, and the amount of bacteria detected was low. The plants produced from the daughter tubers of the CLso positive potato plants were all CLso negative, suggesting that CLso haplotype C was not able to pass to the daughter plants. None of the CLso positive potatoes inoculated in greenhouse or collected from fields showed symptoms characteristic of zebra chip disease, associated with CLso haplotypes A and B.

Key words: '*Candidatus* Liberibacter solanacearum', haplotype C, volunteer potatoes, symptomless infection, grafting, dodder transmission

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Introduction

Gram-negative bacteria belonging to genus *Candidatus* Liberibacter (Jagoueix et al. 1994) are associated with several plant diseases. These bacteria colonize the phloem vessels of plants, and are transmitted by psyllids feeding on the phloem sap. The bacterial species associated with diseases in solanaceous plants, including potato, tomato, pepper and tobacco (Abad et al. 2009; Aquilar et al. 2013; Hansen et al. 2008; Liefting et al. 2009a, 2009b; Lin et al. 2009; Wen et al. 2009) was named *Candidatus* Liberibacter solanacearum (Liefting et al. 2009b) (CLso), syn. *Candidatus* Liberibacter psyllauros (Hansen et al. 2008). Potato zebra chip disease, associated with CLso infection and named after the distinctive striped appearance of the chips prepared of the affected tubers (Secor and Rivera-Varas 2004), has been detected in North and Central America and in New Zealand (Hansen et al. 2008; Liefting et al. 2008, 2009a, 2009b; Lin et al. 2009; Munyaneza et al. 2007; Wen et al. 2009). In addition to the brown discolouration of the vascular ring and the medullary rays in the tubers, the disease symptoms include wilting, pink discolouration, chlorosis and scorching of the leaves, and deformations of the stem (Secor et al. 2009). The zebra chip disease and the other CLso-associated diseases of the solanaceous crop plants are causing severe and increasing crop losses in North and Central America and in New Zealand (Abad et al. 2009; Aquilar et al. 2013; Liefting et al. 2009a; Munyaneza et al. 2007, 2009a, 2009b, 2013). CLso has also been detected in several wild solanaceous plants (Wen et al. 2009) and in the tomato/potato psyllid, *Bactericera cockerelli* Šulc, which is transmitting the bacteria (Hansen et al. 2008; Munyaneza et al. 2007).

In Europe, CLso has not been associated with diseases in solanaceous plants, but with diseases in apiaceous plants, carrot and celery (Alfaro-Fernández et al. 2012; Munyaneza et al. 2010a, 2010b; Teresani et al. 2014). In Finland CLso was repeatedly detected in both the carrot psyllids (*Trioza apicalis* Förster) and the psyllid-infested symptomatic carrots (Haapalainen et al. 2017; Munyaneza et al. 2010a, 2010b; Nissinen et al. 2014). Greenhouse experiments confirmed that the carrot psyllids transmit this bacterium and that the carrot leaf discolouration symptom is associated with a heavy CLso colonization in the plants (Nissinen et al. 2014). After the first discovery in Finland, CLso was subsequently found in symptomatic carrots in Sweden (Munyaneza et al. 2012b), Norway (Munyaneza et al. 2012c), Spain (Alfaro-Fernández et al. 2012), France (Loiseau et al. 2014), and Germany (Munyaneza et al. 2015). In Spain CLso was also detected in symptomatic celery, parsnip and parsley (Alfaro-Fernández et al. 2017; Teresani et al. 2014). In France, CLso was recently detected in the apiaceous crops celery, chervil, fennel, parsley and parsnip, in addition to carrot, and the infected carrot, celery, parsley and chervil plants had severe symptoms (Hajri et al. 2017). While *T. apicalis* is acting as the vector of CLso in northern Europe, in the Mediterranean region the main

vector is *Bactericera trigonica* Hodkinson, feeding on both carrot and celery (Antolinez et al. 2017). CLso was also detected in commercial carrot seed lots in Spain and in seeds collected from infected carrots in France (Bertolini et al. 2014).

By analysing single nucleotide polymorphisms in CLso samples from different locations, Nelson et al. (2011, 2013) found that there are several distinct haplotypes of CLso. Haplotypes A and B occur in the North and Central America (haplotype A also in New Zealand) and are associated with diseases of solanaceous crops. Instead, CLso associated with diseases of apiaceous crops in Europe belong to haplotypes C, D and E (Alfaro-Fernández et al. 2017; Hajri et al. 2017; Nelson et al. 2013; Teresani et al. 2014), of which the haplotype C occurs in northern Europe and the haplotypes D and E in the Mediterranean region. Comparison of the whole genome sequences of different CLso haplotypes revealed that the haplotype C differs in its gene content from both the haplotype A and haplotype B (Wang et al. 2017). There is also sequence variation between different specimens of the same haplotype (Thompson et al. 2015).

Due to the detrimental effect of CLso on potato industry elsewhere, it is an important question whether the European haplotypes of CLso are restricted in their host range to Apiaceae family of plants, or if they can also infect potato. Antolinez et al. (2017) studied the transmission of CLso haplotype E by the psyllids *B. trigonica* and *B. tremblayi*, and detected 3% transmission rate into potato plants with *B. trigonica* and no transmission with *B. tremblayi*. On the other hand, when the transmission of CLso haplotype B into carrot plants by *B. cockerelli* was tested, only 1.5% of the carrots exposed to the tomato/potato psyllid became infected (Munyaneza et al. 2016), suggesting that the risk of disease transmission across the Solanaceae/Apiaceae boundary is small. The aims of this study were i) to find out whether CLso occurs in the field-grown potatoes in the regions where CLso-associated disease is frequent in carrots, and ii) to test whether CLso haplotype C could be transmitted from carrots into potato plants in vitro. In Finland, carrots and potatoes are grown in some areas side-by-side and/or alternating in the crop rotation in the same field plot in different years, resulting in volunteer potatoes growing among the carrot crop. These volunteer potatoes, if becoming infected, could form a pathway for CLso spreading to the potato crop.

Materials and methods

Plant material collected in the field survey

Plant sampling was targeted to the areas considered to have a high probability of CLso infections: the locations where CLso had been previously found in Finland (Munyaneza et al. 2010a, 2010b), and where carrots and potatoes are cultivated adjacent to each other, or in the same crop rotation, resulting

in volunteer potatoes growing among the carrots (Fig. 1a). Especially, samples of volunteer potatoes were collected from psyllid-infested carrot fields with carrots displaying the leaf discolouration symptom typical for CLso infection, and samples of cultivated potatoes were collected at the edges of the fields adjacent to such a carrot field. In 2011-2013, 27 carrot fields and 6 potato fields were included in the survey in Tavastia Proper and Satakunta regions in southwestern Finland. The potato samples were collected in late August or mid-September. In late September 2014, samples of volunteer potato plants were collected from 8 carrot fields located in different regions: Southwest Finland, Tavastia Proper, Satakunta, South Savonia, Ostrobothnia coast and South Ostrobothnia. The collected potato samples included asymptomatic plants, and some plants showing upward rolling or reddish or purplish discolouration in the top leaflets, common symptoms for zebra chip and several other potato diseases. Tissue samples were taken from the potato leaf petioles (in 2011) and stolons and the stolon end of potato tubers (in 2011-13). The samples were stored at -20°C until DNA extraction and subsequent PCR test for the presence of CLso. Daughter tubers from the CLso positive volunteer potato plants, collected from fields in 2012, were planted and grown in the greenhouse, to test if the bacteria could be passed to the new emerging plants. After the PCR test, the daughter tubers were stored for 21-22 weeks at 7 °C, and then planted in fertilized peat-sand mix, one tuber per pot. The temperature in the greenhouse was set at 18°C and the photoperiod to 15 h. The emergence and flowering of the plants was observed and all possible symptoms in the plants were recorded. The plants were harvested 10 weeks after planting. The longest shoot length was measured and tubers were counted and weighed and cut to half for observation of possible zebra chip symptoms, and samples of stolons and stolon ends of the tubers were prepared for PCR analysis.

Transmission experiments in greenhouse with carrot psyllids

A transmission experiment with carrot psyllid colonies infected with CLso haplotype C was performed three times, using a different psyllid pressure on the potato plantlets each time. The carrot psyllids used in the first experiment were originally collected from Haukivuori, eastern Finland, in 2000. They were continuously reared in a greenhouse (L20:D4, 20/15 ºC day/night, 50% RH) at Jokioinen, as described in Nissinen et al. (2007). In the second experiment, new field-collected carrot psyllids from Forssa (F2012) were used in addition to the greenhouse-reared psyllids of colonies originating from Haukivuori (H2000) and Forssa (F2008). In the third experiment, greenhouse-reared carrot psyllids from colonies F2008 and F2012 were used. For 14-18h before starting the plant exposure, the psyllids were starved, i.e. provided no food source but only water. In the first experiment, 10 potato plantlets (cv. Bintje), each confined in a hoop cage, were exposed to 20 adult carrot psyllids per plant for 7 days. In addition, 10 healthy carrot seedlings (cv. Fontana) at the 2-leaf

stage were exposed to 5 carrot psyllid females per seedling for 7 days, to test whether the psyllids were able to successfully transmit CLso into the plants. The psyllid releasing procedure and the cylinder insect cages used for carrots were similar as described in Nissinen et al. (2012b). After the exposure, all the living psyllids were collected from the plants for subsequent DNA extraction and CLso testing. Six weeks later, 0.5 g samples were cut of both the control plants and the psyllid-exposed plants for DNA extraction. In the second experiment, each potato plantlet was confined in an insect cage together with two healthy carrot seedlings. The potatoes had been planted ten days earlier, and carrot seedlings were at the 1-leaf stage. The plants in each cage were exposed to 30 carrot psyllids for 11 days. Four randomly selected psyllids were collected for PCR analysis from each of the insect cages and tested individually, to check that the psyllids were infected with CLso (for further details see Nissinen et al. 2012a). In the third experiment, singly confined potatoes were exposed to the carrot psyllids, starting ten days after planting. Twenty psyllids were released into each cage in three consecutive days, amounting to 60 psyllids in total. Four psyllids were randomly collected from each cage for PCR analysis, and tested individually for the presence of CLso. Two weeks after the release of the first batch of psyllids, the few living individuals found were manually removed, and the plants were sprayed with insecticide to kill any living psyllids possibly remaining. The exposed and control potato plants were grown in the greenhouse (temperature 20/15ºC day/night, photoperiod 20:4 L:D) and observed for possible disease symptom development. After 8 weeks, the daughter tubers were harvested, and samples from stolons and stolon ends of the tubers were prepared for PCR analysis.

Transmission experiments in greenhouse with dodder

To obtain infected source plants, carrot (cv. Fontana) seedlings were infected with CLso by exposure to carrot psyllid feeding, as previously described (Nissinen et al. 2012b). In the first pilot experiment, dodder (*Cuscuta* sp.) seeds were sown beside the growing potato plantlets (cv. Van Gogh) in the same pots. When the dodder had grown and established well on the potato plants, the carrot plants heavily infected by CLso were transferred adjacent to the potato plants. The dodder tendrils were guided to grow around the carrot leaves, to form bridges for CLso transmission (Fig. 1b). Six weeks later the potato stems and dodder tendrils were cut and DNA samples were prepared for PCR analysis. In the second experiment, dodder was first grown in the same pots with alfalfa (*Medicago sativa*) seedlings, and later transplanted onto potato plants (cv. Van Gogh). After four weeks, when the dodder had established on the potato plants, the potatoes were placed next to CLso-infected carrots or uninfected control carrots. In total, there were 18 psyllid-exposed carrot plants showing leaf discolouration symptom and five unexposed carrot plants as controls. The different carrot-potato pairs were

separated from each other by Plexiglass sheets to prevent the dodder tendrils from forming additional bridges to the other plant pairs. After a month, 0.5 g samples were cut from the petioles of both control and psyllid-exposed carrot plants, dodder stems, lowest 5 cm of potato stems and potato tubers, and stored in a freezer (-20ºC) until DNA extraction.

Transmission experiment in greenhouse by grafting

Leaves from CLso-infected symptomatic carrots were grafted in two axils on different stems of four 4-weeks-old potato plants (cv. Van Gogh) (Fig. 1c), and leaves from healthy uninfected carrots were grafted on two potato plants. The grafting sites were wrapped with Parafilm, and the shoots were covered with plastic bags for seven days to prevent wilting of the grafted carrot leaves. Eight weeks after the grafting, the subjected potato stems were cut, and DNA samples were prepared from both the stem base and from a stem section 1 cm under the axil where the graft had been placed. Thus, from each plant four samples were prepared. The underground parts of the plants were left in the soil and stored at 8°C for 4 months, and thereafter samples of 0.2 g pooled stolon fragments from each plant were prepared for DNA extraction. The daughter tubers were stored at 8°C for additional 6 months, and then planted in peat-vermiculite (2:1) mix in 2.0-l pots in the greenhouse. After 18 days, the plants were moved outdoors and replanted in the soil in a test field. Possible symptom emergence was monitored weekly. After 9 weeks, the plants were harvested and the new tubers were weighed, and 0.2 g tissue samples were cut of stem base and stolons for DNA extraction.

Potato exposure experiment in carrot psyllid-infested carrot fields

On 28 May in 2014, healthy minitubers of potato cultivars Arielle, Excellency and Fontane were planted in the area where CLso had been detected in carrots for the first time (Munyaneza et al. 2010a, 2010b). The minitubers were planted in three different carrot fields, near the edge of the field, because the carrot psyllid density is usually highest at the field edges (Nissinen et al. 2000). Minitubers of each of the three cultivars were planted into two carrot rows per field, 10 tubers of each cultivar per row, following a randomized complete block design in which each row formed a block. Thus, in total, 180 minitubers were planted in the carrot rows. The carrots had been sown on 15 May, 26 May and 28 April in the fields one (F1), two (F2) and three (F3), respectively, and the seedlings had not yet emerged in F1 and F2 and were at the cotyledon stage in F3 at the time of potato planting. The potato plants developed from the minitubers were harvested on 10 September at F1, on 16 September at F2, and on 11 August at F3. The tubers were washed, weighed, counted, split and checked for any disease symptoms. DNA was extracted of stolons and stolon ends of the tubers, and presence of CLso was

tested by real-time PCR. During the growing season, carrot psyllid flight was monitored weekly with four sticky traps in each field from the beginning of June to mid-July.

DNA extraction

DNA was extracted of the midribs and petioles of carrot and potato plants and of potato stems, stolons and tuber heel ends using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a minor modification. Prior to DNA extraction, 0.1 g of dissected plant tissue was ground using FastPrep-24 tissue and cell homogenizer (MP Biomedicals, Ohio, USA) in tubes containing Lysing matrix A. In the grafting experiments, 0.2 g plant tissue samples were powdered using Mixer Mill MM400 (Retsch, Haan, Germany), and DNA was extracted by the CTAB method as previously described (Nissinen et al. 2014), except that 2% polyvinyl pyrrolidone 40 was added to the extraction buffer. All the plant DNA samples were eluted in 100 µl of sterile distilled water. Of the carrot psyllids used in the transformation experiments, the DNA of each psyllid was extracted using the Blood and Tissue Kit (Qiagen) and eluted in 30 µl of water. The DNA samples were stored frozen at -20°C.

Detection and quantification of CLso by PCR

Presence of CLso in the plants collected from the fields or from the greenhouse and in the carrot psyllids used in the transmission experiments was tested by PCR with species-specific 16S rRNA gene primers OA2 and OI2c (Table 1). Samples prepared from CLso-free potato and carrot plants were used as the negative controls. The positive control for potato PCR tests was received from USDA, Wapato, USA, and it had been prepared from potato plants exposed to feeding of *B. cockerelli* Šulc harbouring CLso. All the positive findings from potato samples detected at the Natural Resources Institute Finland were verified at the University of Helsinki, by conventional PCR using primer pair OA2/OI2c. Both the DNA extractions and the PCR tests were performed independently in these two laboratories. The CLso haplotype in the positive field potato samples was subsequently determined by PCR with primers LpFrag4-1611F and LpFrag4-480R (Table 1), amplifying a fragment of 16S-23S rRNA intergenic spacer and the 23S rRNA gene, and with primers CL514F and CL514R (Table 1), amplifying the 50S ribosomal protein rplJ/rplL gene region. The PCR reactions, agarose gel electrophoresis and purification of PCR products for sequencing were performed as previously reported (Haapalainen et al. 2017). The DNA samples from the transmission experiments using grafting and dodder-bridging were also tested by nested PCR to detect CLso at a low titre. After the first reaction with the primers OA2 and OI2c, the product was diluted 1/100 in water, and used as the template for the second reaction with primers Lib16S-F and Lib16S-R (Table 1), to amplify a 579

Table 1. PCR primers and probes used for '*Candidatus* Liberibacter solanacearum' (CLso) detection and haplotyping.

Primer or probe	Sequence 5'-3'	Target gene	Reference
OA2	CTCTAAGATTTCGGTTGGTT	CLso 16S	Liefting et al. 2009a
OI2c	TATATCTATCGTTGCACCAG	CLso 16S	Jagoueix et al. 1994
Lib16S-F	TTCTACGGGATAACGCACGG	CLso 16S	Liefting et al. 2009b
Lib16S-R	CGTCAGTATCAGGCCAGTGAG	CLso 16S	Liefting et al. 2009b
LpFrag4-1611F	GGTTGATGGGGTCATTTGAG	CLso 16S-23S	Hansen et al. 2008
LpFrag4-480R	CACGGTACTGGTTCACTATCGGTC	CLso 16S-23S	Hansen et al. 2008
CL514F (rp01F)	CTCTAAGATTTCGGTTGGTT	CLso rplJ/rplL	Liefting et al. 2009b
CL514R (rp01R)	TATATCTATCGTTGCACCAG	CLso rplJ/rplL	Liefting et al. 2009b
LsoF	GTCGAGCGCTTATTTTTAATAGGA	CLso 16S	Li et al. 2009
HLBr	GCGTTATCCCGTAGAAAAAGGTAG	Liberibacter 16S	Li et al. 2006
COXf	GTATGCCACGTCGCATTCCAGA	Plant COX1	Li et al. 2006
COXr	GCCAAAACTGCTAAGGGCATTC	Plant COX1	Li et al. 2006
HLBp[a]	AGACGGGTGAGTAACGCG	Liberibacter 16S	Li et al. 2006
COXp[b]	ATCCAGATGCTTACGCTGG	Plant COX1	Li et al. 2006

[a] Labels: 5′, 6-carboxy-fluorescein (FAM), 3', Black Hole Quencher (BHQ-1)
[b] Labels: 5′, Yakima yellow, 3', Black Hole Quencher (BHQ-1)

bp internal fragment of the 16S rRNA gene. The program for the first PCR was 95 °C for 3 min and 40 cycles of 95 °C 30 s, 65 °C 30 s and 72 °C 60 s, followed by 72 °C for 10 min, and the second PCR program was similar except for the annealing temperature, which was 60 °C. To determine the relative titre of CLso in the positive field potato samples, real-time PCR was run with fluorescent probes and primers specific for CLso 16S and a plant reference gene, mitochondrial cytochrome oxidase (Li et al. 2006 and 2009; Table 1). Both CLso-specific and reference gene reactions were done in triplicates. The 20 µl reactions contained Maxima Probe qPCR mix (Thermo Scientific), 240 nM primers, 120 nM probe and 5 µl of DNA sample, diluted 1/10. PCR program (95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min) was run with Roche LightCycler 480. For comparison, the relative CLso titre was also determined in field-grown carrots. Of the samples collected in 2011, eight DNA samples were randomly picked from each of these three foliar symptom categories: symptomless plants, plants displaying psyllid feeding damage, and plants with both psyllid feeding damage and leaf discolouration symptom (Haapalainen et al. 2017). The real-time PCR of the potato samples from the dodder transmission and the minituber field experiment was run with a different master mix, Premix Ex Taq Probe qPCR master mix (Takara), with the following program: 95 °C for 2 min followed by 45 cycles of 95 °C 10 s and 58 °C 40 s. The two real-time PCR protocols performed equally well in CLso detection. The negative controls were prepared of healthy potatoes grown in Northern Finland, where CLso does not occur (Haapalainen et al. 2017), and the positive controls were CLso haplotype B positive potato samples from USA. Dilution series of one of the CLso positive field potato samples (96) was used for preparing standard curves. The experimentally determined E-values for potato samples were 1.94 and 1.92 for the primer pairs LsoF/HLBr and COXf/COXr, respectively. Relative titre of CLso in the samples was calculated by Pfaffl method (Pfaffl, 2001), to proportion the threshold cycle differences calculated for both CLso 16S and plant COX gene, with the control level for CLso 16S set at value CtLIB=40. Ten-fold dilution series of the plasmid clone of rRNA operon copy B of CLso haplotype C (Wang et al. 2017), mixed with constant 10% of healthy potato DNA, was used as an absolute standard for determining CLso 16S rDNA copy number per reaction.

DNA sequencing

The purified PCR products were sequenced in both directions at the Natural Resources Institute Finland, using an automated sequencer (Applied Biosystems®3500xL Genetic Analyzer Foster City, California, USA). Consensus sequences were created with Sequencher version 5.2.3, and Clustal Omega was used for making sequence alignments.

Results

CLso-infected potato plants found in the fields

Volunteer potatoes were found growing in 13 of the 27 carrot fields inspected in Tavastia Proper and Satakunta regions in 2011-2013 (Table S1). In 2011, one volunteer potato plant (sample 96), and in 2012, 5 potato plants (one cultivated potato at the edge of a carrot field, sample 208, and four volunteer potato plants in a carrot field, samples 224, 230, 272 and 292), and in 2013, three volunteer potato plants in carrot fields (samples 355, 472 and 478) gave positive results in CLso PCR test (Table 2, Fig. 2). Altogether, 11% of the volunteer potato samples tested in 2011-2013 were CLso positive, and the positive samples originated from six different carrot fields. In 2014, none of the 45 volunteer potato samples collected from eight fields at different locations were CLso positive. None of the CLso positive potato plants showed symptoms characteristic of the zebra chip disease, and none of the potato plants showing some symptoms were CLso positive. Several of those CLso negative plants that had curled up leaves were concluded to be infected by *Rhizoctonia solani*, judging from the typical necrotic lesions observed in the underground stem part. The CLso titres, determined by real-time PCR, were relatively low in the stolon samples of the positive potato plants. However, in the samples 96, 230 and 355 the CLso Ct values were at the same level as in the infected potato samples from USA, Pc1 and Pc2, and the relative titre of CLso in the sample 208 from a cultivated potato was similar to the positive control Pc2 (Table 3). When compared to the infected carrots, the relative titres of CLso in the potato samples were low. Especially the carrots showing leaf discolouration symptom had very high titres, on average ten times higher than in the infected carrots without discolouration and over a hundred times higher than in the symptomless infected potatoes (Fig. 3).

Transmission experiments and survival of CLso in potato

Potato plants subjected to feeding by 20, 30 or 60 carrot psyllids per plant in the greenhouse experiments all tested negative for CLso six to eight weeks after the psyllid exposure. In the first experiment, all the psyllids used in the transmission and tested for CLso were positive, and half of the control carrots, each exposed to five psyllids, became infected. In the second experiment, psyllids from three different colonies were used. All the carrots exposed to the field-collected psyllids (F2012) became infected with CLso and showed the discolouration symptom, whereas transmission was not successful with the greenhouse-reared populations, probably due to reduced CLso titre. As previously shown, different carrot psyllid individuals harbour different amounts of CLso, and a successful transmission of CLso into a carrot requires a high titre of CLso in the psyllid (Nissinen et al. 2014). In the third experiment, psyllids derived from two populations in which 80% (F2008) and 94%

Table 2. Occurrence of '*Candidatus* Liberibacter solanacearum' (CLso) in the field samples of potatoes collected in 2011-2013.

	2011		2012		2013			
	Tested plants	CLso-positive	Tested plants	CLso-positive	Tested plants	CLso-positive	Tested plants total	CLso-positive total
Volunteer potatoes (in the carrot fields)	17	1	38	4	18	3	73	8
Main crop potatoes (near the carrot fields)	14	0	44	1	17	0	75	1
Total	31	1	82	5	35	3	148	9

Table 3. Results of '*Candidatus* Liberibacter solanacearum' (CLso) detection from potato DNA samples by real-time PCR, with three negative control potatoes (NC) and two positive controls Pc1 and Pc2.

Samples	CtLIB[a]	CtCOX[a]	Relative CLso titre[b]	CLso 16S copies per reaction[c]
NC1		21.17	1.08	
NC2		21.47	1.32	
NC3		20.49	0.70	
96	28.67	20.95	1741.85	1165.70
208	32.27	22.77	515.27	107.76
224[d]	37.17	23.74	37.25	4.25
230	30.08	22.85	2320.33	459.52
272[d]	36.92	22.96	26.58	5.01
292	33.48	21.88	130.26	48.58
355	29.92	21.44	1040.58	510.72
472	35.31	20.95	21.13	14.48
478	34.71	21.15	35.96	21.62
Pc1	28.37	20.31	1396.90	1414.79
Pc2	30.57	20.97	499.10	331.79

[a] Ct, threshold cycle values for CLso 16S (LIB) and plant COX1 (COX) target genes.

[b] Relative CLso titre calculated by the Pfaffl method (Pfaffl 2001). The zero-reference value =1.

[c] Target gene copy numbers calculated based on the sample CtLIB and the plasmid standard curve.

[d] The CtLIB value of these samples is at the detection limit defined by Li et al. (2009).

Table 4. '*Candidatus* Liberibacter solanacearum' (CLso) transmission from heavily infected carrot plants to recipient potato plants through dodder tendrils.

Experiment	Carrot plant status	Number of potato plants subjected	Number of CLso positive potato plants	
			Stem base samples	Stolon/tuber samples
I	CLso-infected	6	2	nd
II	CLso-infected	18	6	0
II	Healthy	5	0	0

nd, not determined

(F2012) of the individuals were CLso positive were used. Despite of no-choice feeding by 60 carrot psyllids per potato, there was no evidence of CLso transmission into the potato plants. In accordance with the greenhouse test results, all the 180 potato plants experimentally planted in carrot psyllid-infested carrot fields, and grown there for 2.5 to 3.5 months, were found symptomless and CLso negative in real-time PCR, while symptomatic carrots sampled at the time of potato harvest at F1 and F2 were CLso positive. In F3 there were no discolouration symptoms in the carrots, which was in accordance with the very low trap catches of carrot psyllid in that field (Fig. 4).

In the transmission experiments with dodder, one third of the potato plants connected to CLso-infected carrots turned out to be CLso positive after six weeks (experiment I) or four weeks (experiment II) of exposure (Table 4). The positive test results were obtained from the stem base samples of the exposed potato plants, whereas the tuber and stolon samples were negative. The CLso titres were low even in the stem samples, with the Ct values of the positive samples ranging from 34.6 to 37.0. Samples of the dodder tendrils that had been attached to the infected carrots were all CLso positive. The control potato plants that had been connected to non-infected carrots were all CLso negative.

In the grafting experiment, the samples taken eight weeks later from the potato plant stem base tested positive for CLso in three out of the four plants exposed to leaf grafts from CLso-infected carrots. As the grafting sites were approximately 15 cm above the soil level, the positive results indicate that CLso was not only able to survive in the potato stem but also got translocated down to the root collar level. All the eight samples taken from the control plants, grafted with leaves from healthy carrots, were CLso negative. After four months of cold storage, the stolons of one of the four CLso-inoculated plants were CLso positive (Table S2). After the new growing season in the following summer, 15 months after the graft-inoculation, all the daughter plants grown from the 43 tubers of the CLso-inoculated test plants were tested and found CLso negative. No symptoms characteristic of the zebra chip disease were observed in the shoots or tubers of the field-grown daughter plants.

When the daughter tubers of three of the CLso positive field-collected volunteer potatoes were planted into soil after 5 months in the cold storage, plants emerged 14-28 days after planting. All the daughter plants were negative for CLso in the PCR test (Table S3). The shoots did not show any typical zebra chip disease symptoms, nor did the tubers.

Identity of the amplified DNA fragments

The CLso positive samples of potatoes gave PCR products of 1,168 bp, 918 bp, and 673 bp, when amplified with the primer pairs OA2/OI2c, LpFrag4-1611F/LpFrag4-480R, and CL514F/CL514R, respectively. All the PCR products were sequenced. All the 16S rRNA gene sequences showed 100%

identity to the CLso haplotype C sequences previously amplified from carrots and carrot psyllids in Finland (Haapalainen et al. 2017), and 99% identity to the CLso haplotype B DNA, amplified from a potato sample originating from the United States and showing zebra chip symptoms (Wen et al. 2009). The 16S rRNA gene sequences of three positive volunteer potato samples 96, 292 and 355 (collected in 2011, 2012 and in 2013, respectively) were submitted to GenBank (accessions KF170061, KF170064, KJ584934). All the 16S-23S rRNA intergenic spacer and 23S rRNA gene sequences and rplJ/rplL sequences also showed 100% identity to the CLso haplotype C sequences previously amplified from carrots and carrot psyllids in Finland (Nelson et al. 2011; Haapalainen et al. 2017).The 16S-23S rDNA intergenic spacer and 23S rRNA gene sequences and rplJ/rplL sequences of the three positive volunteer potato samples listed above were submitted to GenBank (accessions KJ584926, KJ584929, KJ584932 and MF278355, MF278356, MF278357, respectively).

Discussion

In this study we detected for the first time '*Ca.* Liberibacter solanacearum' haplotype C in field-grown potatoes in Finland. Of these CLso positive potato plants only one was a cultivated potato grown at the edge of a field next to a carrot field and all the others were volunteer potatoes growing within the carrot fields. None of these CLso positive potato plants showed symptoms characteristic of zebra chip disease. However, it is unlikely that the CLso positive PCR results would have arisen from external contamination of the plant samples by e.g. carrot psyllid excretions. The DNA samples of the field-grown potatoes were prepared of the underground stolon and tuber tissues, which could not have been in contact with psyllids. Thus, we conclude that these potato plants, although not displaying disease symptoms, were colonized by CLso. Although the relative titres of CLso in these potato samples were low compared to the high titres detected in the infected carrots (Nissinen et al. 2014, and this study), the real-time PCR Ct values for CLso were comparable to those previously obtained from symptomatic potato plants infected with the American haplotypes of CLso (Li et al. 2009). Previously, symptomless CLso infections have been found by PCR tests in potato plants (Liefting et al. 2008; Pitman et al. 2011) and in tomato plants (Li et al. 2013). A symptomless infection could result from a low inoculum level, or the CLso haplotype C having different interaction with potato than the haplotypes A and B, or the potato cultivars grown in Finland being less susceptible to the disease than the American ones. Alvarado et al. (2012) found that the rate of disease development in potato correlated with the inoculum dose, i.e. the titre of CLso in the potato psyllids to which the plants had been exposed. Thus, our symptomless CLso positive potato plants could be comparable to the ones that received the lowest levels of inoculum. On the other hand, with CLso haplotype C, the

bacterial titre in carrots can be ten times as high as in these infected potatoes, and still the symptoms associated with the bacterial infection are mild. The severe symptoms, suppression of root growth and prominent leaf discolouration correlate with a very high titre of bacteria, CtLIB/CtCOX $>10^5$ (Nissinen et al. 2014), and the discolouration only becomes visible at the late stage of infection.

The amount of CLso bacteria experimentally transmitted to the potato plants through dodder or grafting was also low, and despite the four weeks of exposure to dodder transmission the bacterial colonization did not reach the underground parts of the plants. Thus, it seems that the downward movement of haplotype C in potato phloem was slow compared to the American haplotypes A and B. Levy et al. (2011) detected CLso in some potato stems, other than the one stem inoculated by psyllid-exposure, by week three using real-time PCR and by week four using conventional PCR. This indicated that within three weeks the infection with the American haplotypes of CLso had already become systemic, because the bacteria had to spread through the tubers to reach the other stems (Levy et al. 2011). In the CLso positive volunteer potato plants found in this study the bacteria had reached the underground parts, but the new plants emerging from the daughter tubers were CLso negative. This suggests that the bacteria either did not survive in the tubers over the long cold storage or were otherwise unable to colonize the daughter plants. This apparent inability of CLso haplotype C to be transmitted from tuber to new shoots was probably not caused by the bacteria being unable to survive the temperatures between 5 and 8°C. In carrot roots CLso haplotype C successfully overwintered at similar storage temperatures, and was able to colonise the newly developing leaves the following spring (Haapalainen, unpublished). Moreover, the CLso haplotypes A and B were previously shown to survive in potato tubers over a cold storage period of 6 months and the seedlings grown from these tubers were infected and symptomatic (Rashed et al. 2015). It has been shown that colonization of potato plants by CLso was slower at temperatures from 12 to 17 °C than at 25 to 30 °C (Munyaneza et al. 2012a). However, in southwestern Finland (Jokioinen) the average temperatures during the summer months are quite low, 14.0 ºC, 16.7 ºC and 15.0 ºC in June, July and August, respectively, according to the climatological long-term normal period 1981-2010 (Pirinen et al., 2012). Thus, although the cold climate may be a limiting factor in bacterial multiplication, CLso haplotype C is still capable of colonizing carrot plants and reaching high bacterial densities.

Although the first CLso positive potato plant found was a volunteer in a carrot field heavily infested by carrot psyllids, potato has not been reported as a host plant for the carrot psyllid, and transmission of CLso to potato by carrot psyllids has not been shown in a controlled laboratory environment so far. In cage experiments, the carrot psyllids have been observed to exhibit probing behaviour on potato leaves (Nissinen et al. 2012a), but no eggs were found on the potato plants. Carrot psyllids did not live long on potato: only few *T. apicalis* were found alive on potato after 11 days

(Nissinen et al. 2012a; this study). By contrast, *B. cockerelli* was observed to stay alive on carrots for several weeks (Munyaneza et al. 2016). In an electrical penetration graph (EPG) study, one carrot psyllid individual was observed to produce E wave forms indicating either phloem salivation or ingestion in the potato plant. However, the average duration of the phloem feeding phase was very short, less than 1 min, compared with the 12.2 to 25.7 min spent in the phloem feeding phase on carrot leaves by males and females, respectively (Collins et al. 2014). In comparison, Munyaneza et al. (2016) observed salivation into carrot phloem by three *B. cockerelli* individuals, of which two salivated for 4-5 min and one individual for 2.3 h. If the phloem phase lasts for less than a minute, the carrot psyllid should harbour a very high titre of CLso to be able to transmit the bacteria into a potato plant during such a short phloem contact. Thus, a successful transmission of the bacteria into a potato plant by carrot psyllids is probably a rare event. It could be possible, though, in the carrot fields where the psyllid pressure is very high and the psyllid flight continues for several weeks. The probability of a potato plant being probed by an individual capable of reaching the phloem in a potato leaf and carrying a bacterial titre high enough for infection increases with increasing number of psyllids. However, despite a very high psyllid pressure in one of the carrot fields, none of the 60 experimental potato plants grown from minitubers in that field were infected with CLso by the end of the season 2014. This suggests a low probability of CLso transmission by carrot psyllids even in the field conditions. As a whole, the summer 2014 was not colder than the summers 2011-2013: the accumulated day degrees (DD) above 5 °C from the beginning of the growing season until the end of September at Jokioinen weather station were 1460, 1245, 1467 and 1421 in 2011, 2012, 2013 and 2014, respectively. However, in the second half of June 2014 the weather was exceptionally cold, with some snowfall and night temperatures below zero, which caused a sudden drop in the carrot psyllid flight peak (Fig. 4). This coldness might have affected CLso as well, at the critical moment of transmission. Even if the transmission had happened just before this cold period, the bacteria would have still been in the aerial parts of the potato plants - based on the slow translocation of CLso haplotype C in potato observed in the experiments with dodder - and might thus have been halted by the frost.

A successful transmission may also require the recipient potato plants to be at an early enough growth stage at the time of the psyllid flight. The symptoms associated with CLso infection in potato have been observed to be more severe in plants exposed during the early growth stages (Gao et al. 2009; Rashed et al. 2014). Gao et al. (2009) suggested that the shoot structures of potato plants become less palatable to the psyllid feeding as the plants become more mature. This lowers the probability that the potato crop would become infected with CLso by the carrot psyllids, because at the time of the psyllid flight the cultivated potatoes are already half-way of their growing season.

Meanwhile, the volunteer potatoes that overwinter deep in the soil emerge later than the main crop potatoes and could be more susceptible to psyllid feeding, and thus, to bacterial infection. The CLso-infected volunteer potatoes in carrot fields could serve as inoculum sources, from which some insects capable of feeding on potato phloem could later acquire the bacteria and spread it further to the potato crop. The risk that the commercially produced seed potatoes would become infected is very low, since the High Grade seed potato production area in Finland is in North Ostrobothnia, where CLso has not been detected (Haapalainen et al. 2017).

After our finding of CLso positive potato plants in Finland, several questions concerning the epidemiology of this bacterial pathogen still remain to be answered. First, we do not know how the potato plants got infected in the fields. Second, we do not know if the CLso haplotype C is a pathogen of potato, since none of the infected potato plants showed disease symptoms. Thus far, the potato psyllid *B. cockerelli* is the only psyllid species identified as the vector of potato zebra chip disease (Buchman et al. 2011; Liefting et al. 2008; Lin et al. 2009; Munyaneza et al. 2007; Secor et al. 2009), and this psyllid species does not occur in Europe. Another psyllid in the genus *Bactericera*, *B. nigricornis*, is also able to feed and reproduce on potato (Fathi 2011; Hodkinson 1981), is highly polyphagous (Hodkinson 1981) and occurs in a wide geographic area including Europe. Three *Bactericera* species, *B. trigonica*, *B. tremblayi* and *B. nigricornis*, were found to carry CLso in Spain (Teresani et al. 2015). Of these species, *B. trigonica* was shown to transmit CLso haplotype E into carrot and celery and, at a low frequency (0-3%), into potato plants, whereas *B. tremblayi* failed to transmit the bacteria (Antolinez et al. 2017). *B. trigonica* does not occur in northern Europe, and *B. nigricornis* has only been observed twice in Finland and both these records are old (Aulmann 1913; Lindberg & Ossiannilsson 1960). Recently, Sjölund et al. (2017) found that *Trioza anthrisci* carries CLso haplotype C in Scotland and Sweden. In Finland, *T. anthrisci* is a very common species feeding on cow parsley, *Anthriscus sylvestris*. However, the flight of *T. anthrisci*, which can start already in the end of April if the spring is warm, is very early compared with the potato growing season. In southern Finland, the egg-laying peak of *T. anthrisci* is usually in the end of May or in the beginning of June (Nissinen, personal observation). At that time, potatoes usually have not even emerged yet, which makes it unlikely that *T. anthrisci* would have significance as a vector of CLso on potato. Thus, in Finland, *T. apicalis* is still the most probable candidate for the vector transmitting CLso into potato plants that grow as volunteers within the carrot fields.

The symptoms associated with CLso haplotype C infection in carrot are different from symptoms associated with the haplotypes D and E in the same host plant (Haapalainen et al. 2017). The genetic differences found between the CLso haplotypes A, B and C, especially within the prophage-related regions (Wang et al. 2017), also suggest that they could harbour haplotype or strain

specific effectors that modify the interaction of the bacteria with psyllids and plants. Although the different haplotypes can colonize plants across the Solanaceae and Apiaceae families (Antolinez et al. 2017; Munyaneza et al. 2016; this study), some differences in the interactions may occur due to the adaptation to different psyllid-host plant combinations. However, the natural host plant range of each CLso haplotype in each geographical area might be mainly determined by the host plant range of the available psyllid species that are capable of harbouring and transmitting the bacteria.

Supplementary material

Table S1. Occurrence of psyllid feeding-associated leaf curling symptom, '*Ca*. Liberibacter solanacearum' (CLso) and the associated leaf discolouration symptom in the inspected carrot fields.

Table S2. '*Ca*. Liberibacter solanacearum' (CLso) test results and number of viable tubers of the potato plants inoculated in greenhouse by grafting with leaves from CLso-infected carrot plants (test) and healthy carrot plants (control).

Table S3. Number and viability of the tubers and health of the daughter plants of '*Ca*. Liberibacter solanacearum' (CLso) positive volunteer potato plants found in carrot fields in 2012. The tubers were stored over winter at +7 °C before sprouting.

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Fig. 1 Natural and experimental transmission of '*Candidatus* Liberibacter solanacearum' haplotype C into potato plants. a) A volunteer potato plant growing in a carrot field, in the middle of carrot plants that display the leaf discolouration symptom associated with CLso infection. b) Transmission of CLso by dodder that forms a bridge between a potato plant and an infected carrot plant. c) Transmission of CLso into potato plants by grafting with leaves of infected carrot plants

Fig. 2 DNA samples from carrots, potatoes and carrot psyllids gave positive results for '*Candidatus* Liberibacter solanacearum' in PCR. In the upper gel, PCR products amplified with primers OA2 and OI2c (1168 bp), and in the lower gel, with primers LpFrag4-1611F and LpFrag4-480R (918 bp). Lanes 1 and 13, GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific), and lanes 2-12, PCR products: 2, potato 96 (2011); 3, carrot 117 (2011); 4, psyllid 12/17 (2011); 5, potato 292 (2012): 6, carrot 301 (2012); 7, psyllid 302 (2012); 8, potato 355 (2013), 9, potato 472 (2013); 10, positive control 170112 USA; 11, healthy potato; 12, water control. These PCR products were subsequently sequenced

Fig. 3 The titre of '*Candidatus* Liberibacter solanacearum' haplotype C was low in the positive potato plants compared to infected carrots. The relative CLso titre in the field-grown plants was calculated from the real-time PCR results by the Pfaffl method. NS, no symptoms; PD, psyllid damage; PD+C, psyllid damage and discolouration. For each symptom category of carrots n=8, and for CLso positive potatoes n=9. The average of the relative titres is shown above each bar. Error bars indicate standard deviation

Fig. 4 Weekly sticky trap catches of the carrot psyllid, *Trioza apicalis*, in the three carrot fields where potato minitubers were planted to study natural transmission of '*Candidatus* Liberibacter solanacearum'. The trap catches per week are shown as averages of 4 traps in each field (F1, F2, F3). Error bars indicate standard deviation

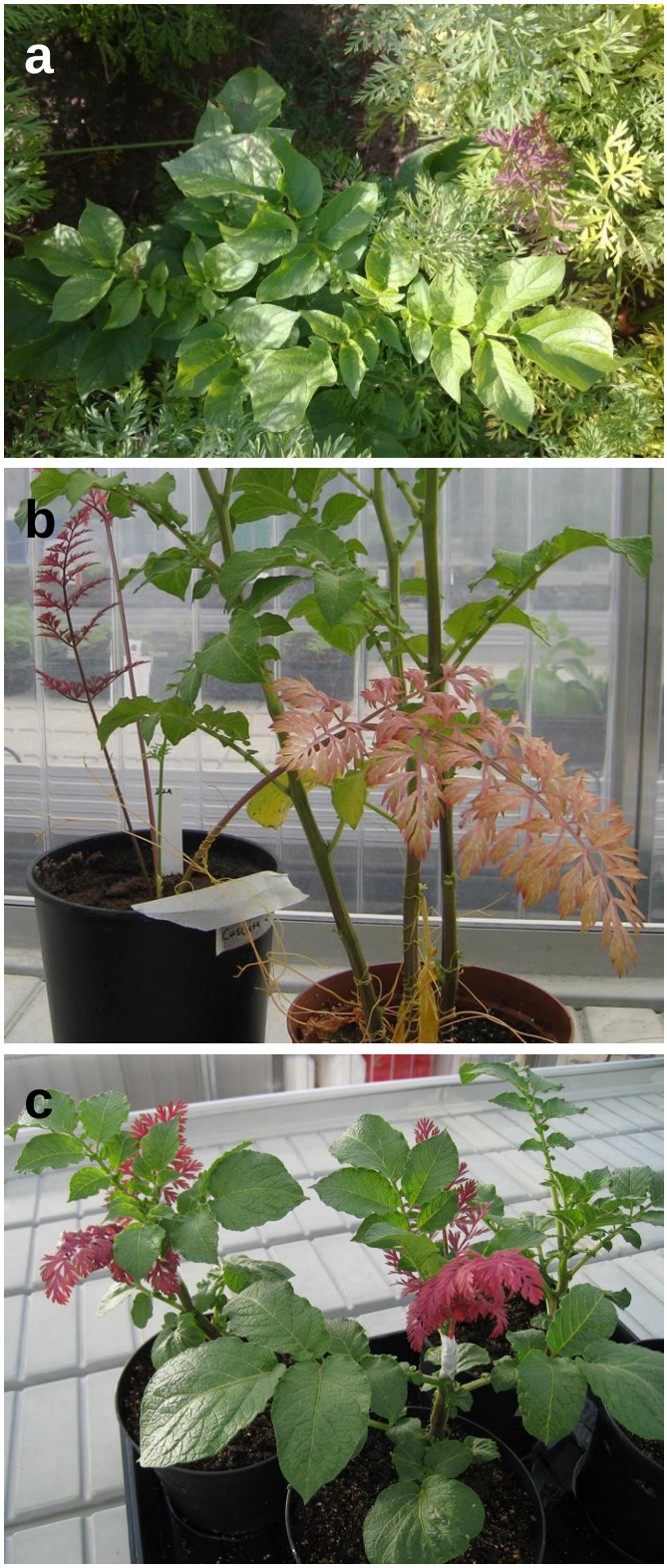
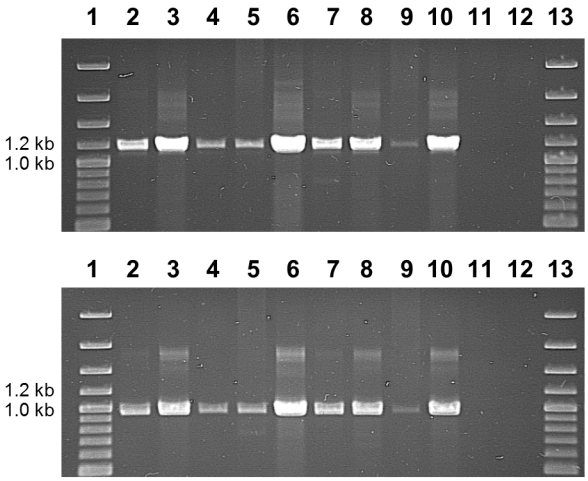


Fig.1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1.2 kb
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Fig. 2

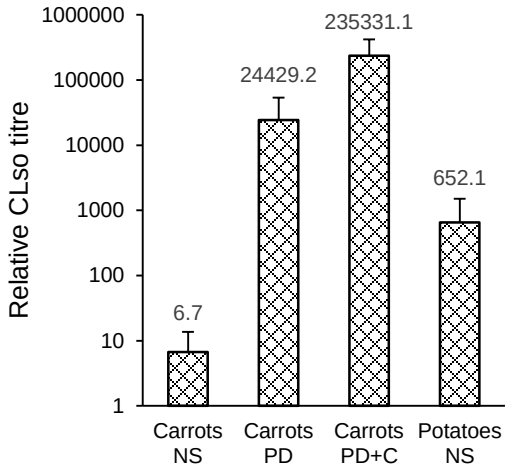
Relative CLso titre
1000000
100000
10000
1000
100
10
1
6.7
24429.2
235331.1
652.1
Carrots NS
Carrots PD
Carrots PD+C
Potatoes NS

Fig. 3

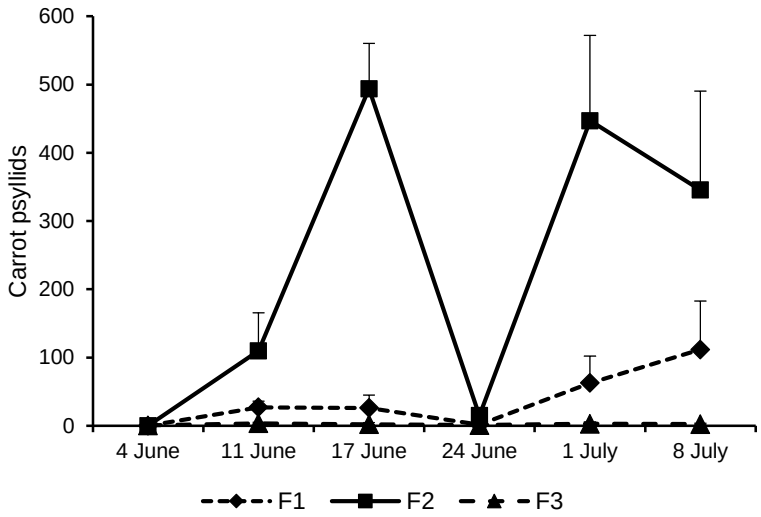
600
500
400
300
200
100
0
Carrot psyllids
4 June
11 June
17 June
24 June
1 July
8 July
F1
F2
F3

Fig. 4

Supplementary material

Table S1. Occurrence of psyllid feeding-associated leaf curling symptom, '*Ca*. Liberibacter solanacearum' (CLso) and the associated leaf discolouration symptom in the inspected carrot fields.

Carrot foliar symptoms		Asymptomatic		Leaf-curling		Leaf-curling and discolouration	
Year	Field code	Plants[a] (%)	CLso positive[b] (%)	Plants[a] (%)	CLso positive[b] (%)	Plants[a] (%)	CLso positive[b] (%)
2011	1	62	0	32	20	0[c]	82[c]
	2	61	0	29	80	7	100
	3	nd	0	nd	100	nd	100
	4	48	50	26	80	24	100
	5	47	20	47	40	7	100
	6	1	100	30	100	70	100
	7*[d]	11	100	35	80	55	100
	13	21	67	40	80	39	100
	14	42	50	36	40	22	100
	15	14	60	47	100	39	100
2011 average		34.1	40.7	35.8	70.8	29.2	97.4
2012	19	6		73		21	
	20*	19		58		24	
	21*	12		63		25	
	23	4		53		43	
	24	14		81		6	
	27	7		80		14	
	28*	2		56		42	
	29	33		56		10	
2012 average		12.1	nd[e]	65.0	nd	23.1	100.0
2013	31	13		34		53	
	32	76		18		7	
	33*	59		23		18	
	34	20		38		42	
	35	49		25		26	
	37*	27		11		61	
2013 average		40.7	nd	24.8	nd	34.5	85.7

a Proportion of plants classified in each symptom category. Plant symptoms were visually inspected of 200 plants diagonally throughout the field.
b CLso was tested by conventional PCR of a subsample of plants from each symptom category (Haapalainen et al. 2017).
c Although none of the 200 plants systematically inspected showed discolouration symptoms, some plants in the field were symptomatic and were sampled.
d *= The plots from which the CLso-positive potato samples were found
e nd= not defined

Supplementary material

Table S2. '*Ca*. Liberibacter solanacearum' (CLso) test results and number of viable tubers of the potato plants inoculated in greenhouse by grafting with leaves from CLso-infected carrot plants (test) and healthy carrot plants (control).

Potato plant	Positive CLso tests[a] Stem upper	Positive CLso tests[a] Stem base	Stolon CLso test[b]	Number of viable tubers (producing daughter plants)
1 (control)	0/2	0/2	negative	6
2 (control)	0/2	0/2	negative	9
3 (test)	1/2	1/2	negative	6
4 (test)	0/2	0/2	negative	6
5 (test)	1/2	1/2	positive	9
6 (test)	0/2	2/2	negative	7

[a] The potato plant stems were cut and tested for CLso infection 8 weeks after inoculation.
[b] The stolons were tested after four months of storage at 8°C.

Supplementary material

Table S3. Number and viability of the tubers and health of the daughter plants of '*Ca*. Liberibacter solanacearum' (CLso) positive volunteer potato plants found in carrot fields in 2012. The tubers were stored over winter at +7 °C before sprouting.

Potato plant sample code	Potato plant CLso test	Number of tubers	Number of emerged plants	Daughter plant CLso test
224	positive	5	5	all negative
272	positive	2	2	all negative
292	positive	6	3	all negative
304	negative (control)	11	4	all negative